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# **Role of PIEZO Channels in Ultrasound-stimulated Dental Stem Cells**

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## **Abstract**

Piezo1 and Piezo2 are mechanosensitive membrane ion channels. We hypothesized that Piezo proteins may play a role in transducing ultrasound-associated mechanical signals and activate downstream MAPK signaling processes in dental cells. In this study, the expression and role of Piezo channels were investigated in dental pulp stem cells (DPSC) and periodontal ligament stem cells (PDLSC) following treatment with low-intensity pulsed ultrasound (LIPUS). Cell proliferation was evaluated by BrdU incorporation. Western blots were used to analyze the proliferation-associated marker PCNA as well as the transcription factors, c-fos and c-jun. ELISA and Western blotting were used to determine MAPKs activation following LIPUS treatment. Ruthenium red (RR), a Piezo ion channel blocker, was applied to determine the functional role of Piezo proteins in LIPUS-stimulated cell proliferation and MAPK signaling. Western blotting demonstrated the presence of Piezo1 and Piezo2 in both dental cell types. LIPUS treatment significantly increased the level of the Piezo proteins in DPSC after 24h, however no significant effects were observed in PDLSC. Treatment with RR significantly inhibited LIPUS-stimulated DPSC proliferation, but not PDLSC proliferation. ERK1/2 MAPK was consistently activated in DPSC over a 24h time period following LIPUS exposure, whereas phosphorylated JNK and p38 MAPK were mainly increased in PDLSC. RR affected MAPK signaling in both dental cell types with its most prominent effects on ERK1/2 MAPK phosphorylation levels; the significant inhibition of LIPUS-induced stimulation of ERK1/2 activation in DPSC by RR suggesting that stimulation of DPSC proliferation by LIPUS involves Piezo-mediated regulation of ERK1/2 MAPK signaling. This study for the first time supports the role of Piezo ion channels in transducing the LIPUS response in dental stem cells.

**Key words:** Dental pulp cells, periodontal cells, Piezo1, Piezo2, MAPK, ultrasound.

## Introduction

Following the recognition of low intensity pulsed ultrasound (LIPUS) as a stimulant to promote bone fracture healing (1, 2), LIPUS is now also increasingly regarded as a potential adjuvant therapy in stem cell-based tissue engineering (3-6). Studies have demonstrated LIPUS enhanced viability, proliferation and multilineage differentiation in a variety of postnatal mesenchymal stem cell (MSC) populations and the efficacy may be modulated by signaling pathways such as ERK1/2 and p38 MAPK (3, 7, 8). Our previous study highlighted that proliferation of dental pulp stem cells (DPSC) and periodontal ligament-derived stem cells (PDLSC) was promoted by LIPUS via distinct MAPK signaling pathways (9). ERK1/2 played a critical role in LIPUS induced DPSC proliferation whilst p38 and JNK were essential in LIPUS-stimulated PDLSC proliferation. However, the early cellular biomechanical responses and upstream factors necessary for MAPKs activation by LIPUS are yet to be elucidated.

MSC, including BMSC, DPSC and PDLSC, are mechanosensitive and play an important role in tissue homeostasis and repair. It is understood that LIPUS generates mechanical stresses which can affect specific cellular mechanical transduction components, such as integrins, focal adhesion complexes, membrane receptors, ion channels and cytoskeleton components (10). Notably following exposure to mechanical stimuli, mechano-sensitive ion channels are known to be able to induce downstream signaling processes which eventually lead to change in cellular behavior (11).

Piezo1 and -2 proteins are transmembrane pore-forming cation channels which have recently been identified as being mechanoresponsive in numerous eukaryotic cell types (12-15). Thus Piezo proteins represent mechanically-activated (MA) channels inducing cationic currents across the cell membrane (12, 13). Their role in cellular migration, proliferation, and elongation has implicated a functional relationship of Piezo proteins with integrin activation (16, 17). Piezo1 and -2 are encoded by FAM38A and FAM38B genes, respectively and they exhibit different expression profiles (18). Unlike Piezo1, Piezo2 is particularly highly expressed in dorsal root ganglia suggesting involvement in neural system responses (16). Based on previous data, we hypothesised that Piezo-1 and -2 may be important cell membrane-located

mechanotransduction components expressed on dental cells and involved in activating intracellular signaling which underpins cellular responses. In this study, we therefore investigated the presence of Piezo1 and -2 in DPSC and PDLSC, and their involvement in LIPUS-associated proliferation and MAPK signaling.

## **Materials and Methods**

### *Cell Culture*

DPSCs were obtained from pulp tissue of incisors and PDLSC from periodontal ligament of molars from 6-weeks old male Wister Hann rats (weight 120g; Charles River Laboratories, via Aston University, Birmingham, UK) as described previously (9, 19, 20). In brief, to isolate DPSC, pulp tissue was minced into pieces of  $\sim 1 \text{ mm}^3$  and digested at 37 °C with 0.25 % (w/v) trypsin and 1 mM EDTA (Gibco, Paisley, UK) for 30 min. After centrifugation at 900g for 5 min, the cell pellet was re-suspended and seeded in  $\alpha$ MEM/20 % FBS. For PDLSC isolation, the extracted periodontal tissue was incubated with 0.25 % (w/v) trypsin and 1 mM EDTA (Gibco, Paisley, UK) for 45 min at 37 °C. Cell pellets were re-suspended and seeded in  $\alpha$ MEM/20 % FBS. The established DPSC and PDLSC cultures, used for the experiments at passages 3-5, were shown to express a range of stem cell markers and had multilineage differentiation potential (9).

### *LIPUS Treatment*

A calibrated therapeutic ultrasound device (DuoSon, SRA Developments, UK) emitting pulsed ultrasound (63-Hz repetition rate with 3.2 milliseconds pulse duration) at a frequency of 1 MHz was used. An in-house custom built silicon anti-reflection chamber to house 6-well culture plates was used for ultrasound irradiation (21, 22).

One-day prior to ultrasound treatment, 10,000 cells were seeded into each well containing  $\alpha$ MEM / 10% FBS. Cells were washed and 4mL fresh supplemented culture medium was added to each culture dish immediately prior to ultrasound exposure. The following ultrasound

parameters were applied based on our previous experience (9): 1 MHz 250 mW/cm<sup>2</sup> for DPSC and 1 MHz 750mW/cm<sup>2</sup> ultrasound was used for PDLSC for 5 min.

In Ruthenium Red (RR, Sigma, St. Louis, MO) inhibition experiments, RR was freshly diluted with ddH<sub>2</sub>O and 20 µM RR was added to cultures 5 min prior to ultrasound treatment (e.g. 13).

### *Western Blotting*

Cells were removed from the culture plastic in ice-cold PBS using a cell scraper (Millipore, UK). After centrifugation, the cell pellet was lysed in RIPA buffer (Sigma-Aldrich, UK) and analyzed using the Bradford assay. Twenty-µg of protein was electrophoresed on pre-cast 4–12% Bis-Tris gels (Invitrogen). Protein was transferred from gels to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot® transfer system (BIO-RAD). Membranes were incubated with 5% fat-free milk prior to incubation with rabbit polyclonal antibodies directed against GAPDH (1:10000 Abcam), FAM38A/Piezo1 (1:500 Abcam), FAM38B/Piezo2 (1:500 Abcam), and c-fos (1:1000 Cell Signaling Technology), c-jun (1:1000 Cell Signaling Technology), PCNA (1:1000 Cell Signaling Technology), ERK (1:1000 Cell Signaling Technology), phospho-ERK (1:1000 Cell Signaling Technology), p38 MAPK (1:1000 Cell Signaling Technology), phospho-p38 MAPK (1:1000 Cell Signaling Technology), JNK (1:1000 Cell Signaling Technology), phospho-JNK (1:1000 Cell Signaling Technology) overnight at 4°C. After washing with PBS, Goat anti-rabbit IgG (680 RD, LI-COR Biosciences) was applied for 90 minutes at room temperature (1:5000, LI-COR). Visualization and quantification was performed using the LI-COR Odyssey® scanner and software (LI-COR Biosciences). Quantification was undertaken using ImageJ software (23). Expression of Piezo1/2, PCNA, c-fos and c-jun are presented as GAPDH normalized ratio calculated as Target Protein (PCNA/c-fos/c-jun)/GAPDH.

## *ELISA*

Specific ELISAs were used to quantify total and phosphorylated p38, ERK1/2, JNK p38 MAP kinase: ERK 1/2 ELISA Kit SimpleStep (ab176660), p38 MAPK alpha ELISA Kit SimpleStep (ab176664), JNK 1/2 ELISA Kit SimpleStep (ab176662) (all from Abcam, Cambridge, UK). Whole cell extracts were collected from cultures following manufacturer's instructions. The phosphorylated ratio of MAPK pathway proteins was calculated as phospho-MAPK/ total MAPK and used as a parameter of pathway activation as previously described (9).

## *Cell Proliferation Analysis*

Cell proliferation was evaluated by BrdU incorporation using a commercial cytochemical assay kit (Roche Diagnostics GmbH, Mannheim, Germany) (9). Cultures were labelled using 10  $\mu$ M BrdU label for 1 h, rinsed with PBS and fixed in ethanol for 20 min, followed by immunolabelling using BrdU antibody. Images were captured using an AxioCam phase-contrast microscope (Zeiss, UK) and analyzed with Image J software (9, 23). Cell counts are presented as averages from 5 fields per time-point from at least three independent experiments.

## *Statistical Analysis*

All data are expressed as the mean  $\pm$  standard deviation (SD) and statistically analyzed using SPSS 10.0 for Windows (SPSS Inc., USA) using one-way analysis of variance for comparison between the control and test groups. A value of  $P < 0.05$  was considered statistically significant.

## Results

### *LIPUS Increased Piezo1 and Piezo2 in DPSC*

The protein expression of Piezo1 and -2 in the dental stem cells was evaluated by Western-blot analysis. Both Piezo proteins were evident in DPSC and PDLSC (Fig. 1). Interestingly, LIPUS significantly increased expression of Piezo1 and Piezo-2 in DPSC at 24h after treatment. A modest increase in Piezo1 and decrease of Piezo2, both non-significantly, were observed in PDLSC (Fig. 1).

### *LIPUS Stimulated Cell Proliferation is Inhibited by the Piezo Protein Blocker, Ruthenium Red*

LIPUS stimulated proliferation of DPSC and PDLSC was evidenced by enhanced BrdU incorporation and underlined by increased expression of the proliferation associated protein, PCNA (Fig. 2A, B, D, E). Moreover, the expression of the transcription factors, c-fos and c-jun, were increased in both dental stem cell types following LIPUS treatment (Fig. 2A, B, D, E). The highest levels of PCNA and c-jun were observed 24 h (Fig. 2 B, E) whilst c-fos reached peak levels at 4 h post-LIPUS treatment (Fig. 2 B,E).

Inclusion of Ruthenium Red (RR), a pharmacological blocker of Piezo channels (13), 5-min prior to LIPUS treatment, resulted in significant inhibition of LIPUS-stimulated proliferation of DPSC in comparison with control cultures (Fig. 2C). However, in PDLSC cultures, the LIPUS-stimulated proliferation was not affected by RR (Fig. 2F). These data suggest that Piezo signaling was involved in LIPUS effects on DPSC, but not on PDLSC proliferation.

### *Role of Piezo Proteins in LIPUS Activated MAPKs*

Recently, we reported that ERK1/2 phosphorylation was associated with LIPUS-stimulated proliferation of DPSC, whilst JNK and p38 were selectively involved in PDLSC responses (9). Here



using Western blot analysis, the results indicate a persistent increase in phosphorylated ERK1/2 of up to 24h in DPSC (Fig. 3). In both dental stem cells, JNK activation was evident at 4h after LIPUS, whilst it was significantly decreased at 24h post-treatment in DPSC; p38 MAPK was significantly decreased in DPSC but was increased in PDLSC 24h after LIPUS (Fig. 3). These findings confirm the distinct MAPK activation profiles induced by LIPUS in different dental stem cell types.

To analyse the potential role of Piezo proteins in the LIPUS-induced activation of MAPK signaling, ELISAs were performed. As RR is known to only exert transient blocking effect on these ion channels, and to highlight the immediate and direct effect of RR/Piezo inhibition on MAPK signaling, MAPK phosphorylation was studied at time points relatively shortly after LIPUS treatment (5 min and 1h).

The results demonstrate that LIPUS-induced ERK1/2 phosphorylation in DPSC was significantly inhibited by RR by ~75% (Fig. 4A). Similarly, the decreased phosphorylated ERK1/2 levels were further diminished by RR treatment in the PDLSC cultures (Fig. 4D). RR also reduced LIPUS-stimulated phosphorylated JNK in PDLSC and p38 MAPK levels at 1h post-LIPUS in DPSC and PDLSC (Fig. 4 B-F).

## **Discussion**

LIPUS is an emerging but as yet undeveloped therapy for dental tissue repair (24). Previously we were able to show that LIPUS stimulated proliferation and MAPK signaling in different dental stem cell types. Notably proliferation of DPSC appeared dependent on ERK1/2 MAPK activation, whereas stimulation of PDLSC was associated with JNK and p38 MAPK pathways (9).

This study was designed on the premise that LIPUS effects on dental cells were mediated by initial interchanges at the cell membrane leading to subsequent intracellular activation via MAPK transduction pathways. In particular, we were interested in determining whether mechano-sensitive Piezo ion channels are involved in the mechanisms underlying LIPUS effects (10-12). Subsequently, this study demonstrated the presence of Piezo proteins in dental stem

cells and is the first to implicate Piezo1 and -2 in LIPUS signaling. Others have shown that Piezo proteins are widely expressed with reportedly the highest expression in kidney and lung tissues (17). In human periodontal ligament (PDL) cells, Piezo-1 activation was shown to be associated with NF- $\kappa$ B signaling and was proposed to be involved in mechanical stress-induced osteoclastogenesis (25). Interestingly, in this study the expression of Piezo1 and Piezo-2 proteins were both increased in DPSC after LIPUS treatment, but no significant changes were observed in PDLSC. Preliminary findings also indicated enhanced Piezo1 gene expression in DPSC following LIPUS treatment (unpublished data). Further research is warranted to study further the short- and long-term effects of LIPUS on the expression of Piezo channels and to elucidate the processes involved in the cell-specific LIPUS-induced increase in Piezo protein expression which may reflect the responsiveness of the Piezo membrane ion channels to LIPUS.

To clarify the role of Piezo proteins, we utilized RR, a polycationic pore blocker, which is known to inhibit the mechanosensitive Piezo1 and Piezo2 membrane channels (13). By blocking the transmembrane Piezo proteins, RR inhibits the Piezo-induced mechanically-activated (MA) currents from the extracellular surface and reportedly exerts its maximum inhibition 5 to 20 min prior to stimulation (26). Results indicated that RR abolished LIPUS-promoted DPSC proliferation, but had no effect on PDLSC, underscoring cell-specific involvement of Piezo proteins after LIPUS stimulation. These findings also indicate that Piezo proteins are related to particular LIPUS-induced signaling transduction processes (9). Indeed, following analysis of the MAPK pathways, this study indicated that ERK1/2 in particular was consistently activated in DPSC following LIPUS treatment; a process which was significantly affected by the RR Piezo channel blocker. It is unclear why RR reduced phosphorylated ERK1/2 levels in the LIPUS-treated cells to below control levels; it may be speculated that these data reflect differential, time-dependent dynamics of the MAPK (de)-phosphorylation processes. As ERK1/2 signaling was identified previously as a pivotal pathway in LIPUS stimulation of DPSC proliferation (9), these data highlight that Piezo-dependent stimulation of ERK1/2 phosphorylation is involved in promoting DPSC proliferation following LIPUS treatment (Fig. 2C; 9). Considering the complex interplay of various membrane receptors/channels and transduction signaling cascades, it is plausible that other mechanisms than the proposed Piezo/ERK1/2 pathway may be involved in

LIPUS stimulation of DPSC. The precise complex biophysical interaction of LIPUS with cells is still not fully understood and the biological response may well be dependent on subtle changes of the ultrasound characteristics such as frequency and intensity. Potentially the mechanical microstreaming induced by LIPUS impacts the cell membrane of DPSC resulting in the opening of the Piezo channels with subsequent transport of cations leading to MAPK activation (15). This notion corresponds with the well-established capacity of ultrasound to create pores in the cell membrane by sonoporation thereby enhancing cross membrane transport (recently reviewed in 27).

The Piezo association with ERK1/2 was also implicated for PDLSC, although this relationship involving a decreased ERK1/2 in LIPUS/RR-treated PDLSC could not account for LIPUS stimulation of PDLSC proliferation (Fig. 2; 9). This finding confirmed that RR at the concentration used was effective in modulating cell signaling pathways in the PDLSCs. Moreover, the finding that RR also appeared to interfere with not only ERK1/2, but also JNK and p38 MAPK pathways in PDLSC highlight that Piezo cation membrane channels participate in wide-ranging signaling processes (16, 17). More work is required to elucidate the precise role of the Piezo channels by selective inhibition, e.g. by using Piezo1 or Piezo2 specific siRNAs. Furthermore, it would be interesting to investigate the role of other mechanosensitive cation channels in the biophysical signal transduction of LIPUS, for example by studying the effects of the gadolinium, a known pharmacological blocker of stretch-activated non-selective cation channels (28). Further study is also warranted to unravel the mechanisms underlying the distinct dental cell type-specific signaling responses to LIPUS. Intrinsic differences between DPSC and PDLSC may be related to their distinct niche origins, possibly involving mechanical (de)sensitization (9), and/or their different spatial embryonic origins during tooth development (DPSC, dental papilla; PDLSC, dental follicle).

In conclusion, this study has demonstrated the presence of the membrane ion channels Piezo1 and Piezo2 in dental stem cells. These mechanosensitive membrane cation channels were implicated for the first time as being involved in LIPUS-mediated stimulation of DPSC proliferation, at least in part through ERK1/2 MAPK signaling (9). Piezo-1 and, -2 expression and

MAPK activation were also highlighted in PDLSC, but were apparently not associated with LIPUS stimulation of PDLSC proliferation further underscoring cell type-specific effects of LIPUS (9). Further research is warranted to address the specific roles of Piezo1 and Piezo2 membrane channels in the stimulation of MAPK signaling and cell proliferation. Clinically, LIPUS is considered as an effective non-invasive therapeutic tool to enhance hard tissue repair and fracture healing. This study further supports the potential of LIPUS to promote stem cell-based dental tissue healing. Application of LIPUS to boost self-repair processes of dental tissues is an exciting field requiring further basic and (pre)clinical studies.

### **Author Contributions**

Qianhua Gao contributed to conception, design, data acquisition, data analysis and interpretation, manuscript writing; Paul Cooper and Damien Walmsley, contributed to conception, design, data interpretation, and critically reviewed the manuscript; Ben Scheven contributed to conception, design, data analysis, interpretation and writing of the manuscript. All authors gave final approval of the manuscript.

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## Figure Legends:

**Figure 1.** Piezo1 and Piezo2 expressions in dental stem cell types. A and C: Immunoblotting images of control or LIPUS-treated DPSC and PDLSC and processed for Western blot analysis 4 h and 24h post-treatment. B and D: Quantitative analysis of protein levels of Piezo-1, -2 normalized against GAPDH. \* $P < 0.05$  and \*\*\* $P < 0.001$  versus the untreated controls. Values represent the mean  $\pm$  SD of three independent assays.

**Figure 2.** LIPUS stimulates dental stem cell proliferation. A and D: Western-blotting images of proliferation-related proteins PCNA, c-fos and c-jun in DPSC and PDLSC treated by LIPUS. B and E: colorimetric analysis and quantification of PCNA, c-fos and c-jun protein levels. C and F: Cell proliferation as assessed by BrdU incorporation analysis (with or without prior LIPUS and/or Ruthenium Red (RR) treatment); C: RR decreases proliferation in LIPUS-treated DPSC group. F: RR did not affect proliferation in LIPUS-treated PDLSC group. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus the untreated controls. # $P < 0.05$  versus the experiments. Values represent the mean  $\pm$  SD of three independent assays.

**Figure 3.** Western blot analysis of MAPK proteins in dental stem cells 4 and 24h following LIPUS treatment. LIPUS affected the phosphorylated levels of MAPKs in DPSC and in PDLSC. A: Immunoblots of DPSC and PDLSC subjected to LIPUS for 5 min. B – F: Quantitative changes of MAPK levels in DPSC and PDLSC. The phosphorylated ratio MAPKs are presented as a parameter of MAPK activation. Phosphorylated ratio = phospho-MAPKs / total MAPKs. \* $P < 0.01$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus the untreated controls. Values represent the mean  $\pm$  SD of three independent assays.



**Figure 4.** Effects of the Piezo inhibitor Ruthenium red (RR) on MAPK phosphorylation patterns after LIPUS treatment of DPSC and PDLSC. Experimental groups were treated with 20  $\mu$ M RR and 5 min LIPUS and incubated for 5 min and 1h post-LIPUS treatment. Activation of MAPKs were quantitatively analyzed by phospho-MAPKs/total MAPKs (phosphorylated ratio). A-C: Change of ERK1/2, JNK and p38 MAPK activation with/without LIPUS and RR in DPSC. D-F: Change of ERK1/2, JNK and p38 MAPK activation with/without LIPUS and RR in PDLSC. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus the untreated controls; ++ $P < 0.01$ , +++ $P < 0.001$  versus corresponding LIPUS treated groups # $P < 0.05$ , ### $P < 0.001$  versus RR controls. Values represent the mean  $\pm$  SD of three independent assays.

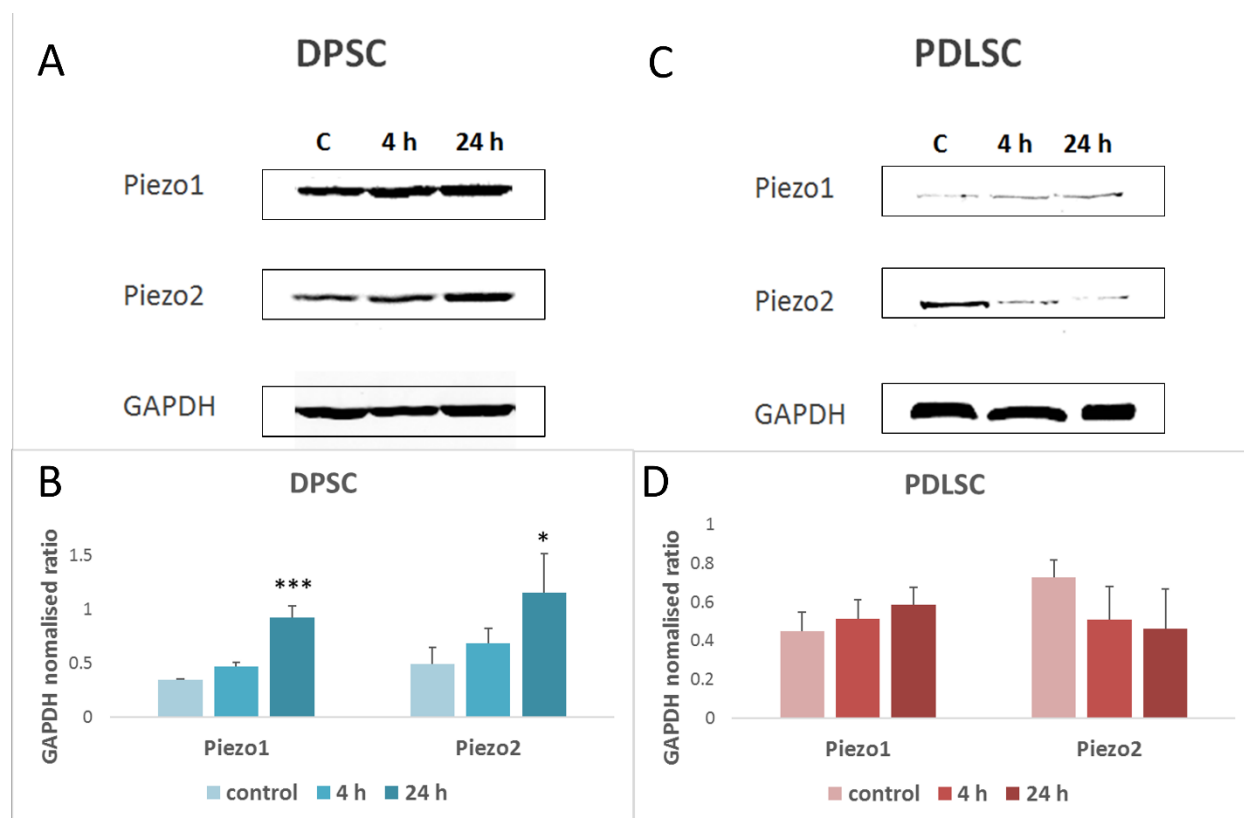


Fig. 1

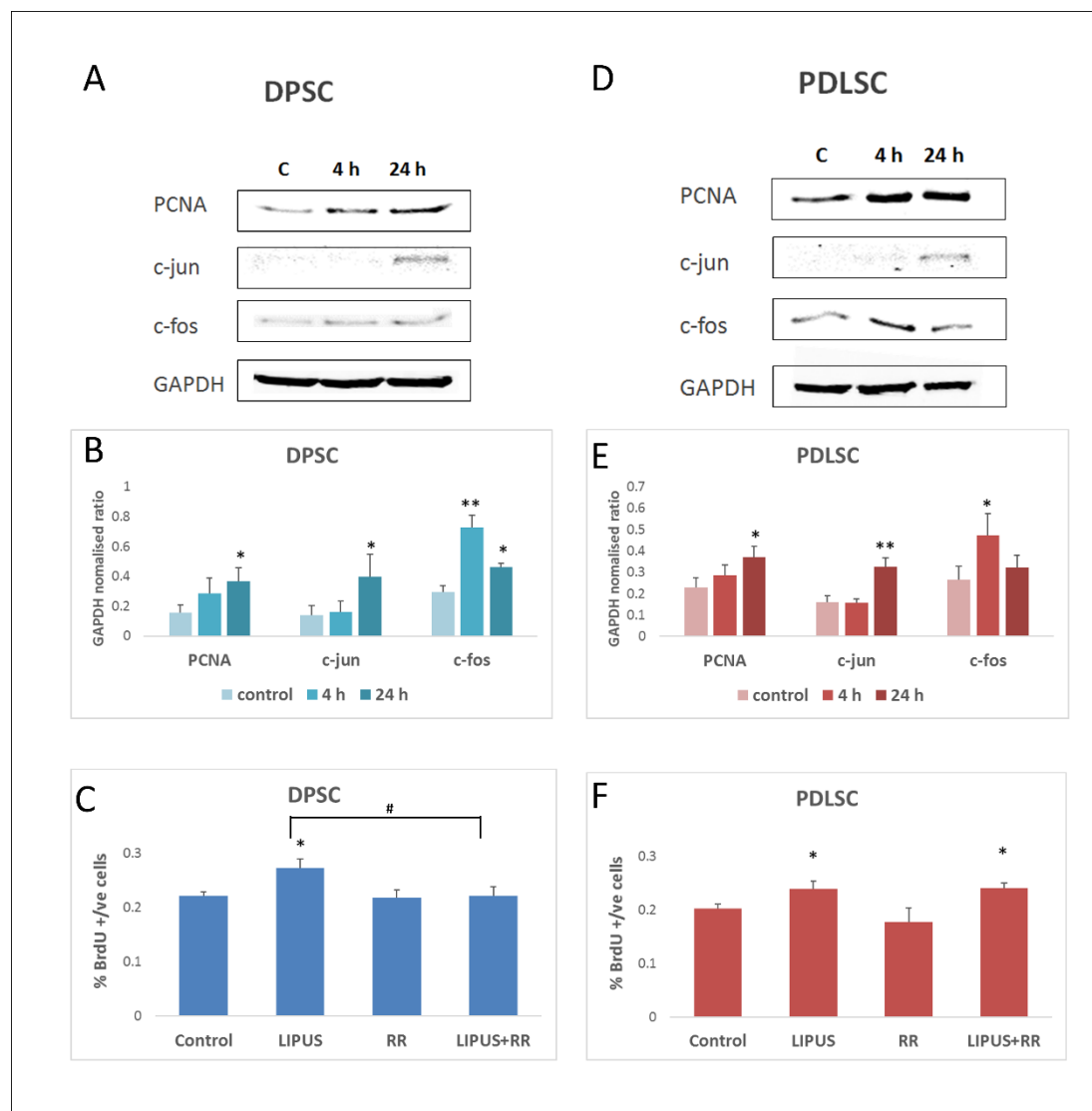


Fig. 2

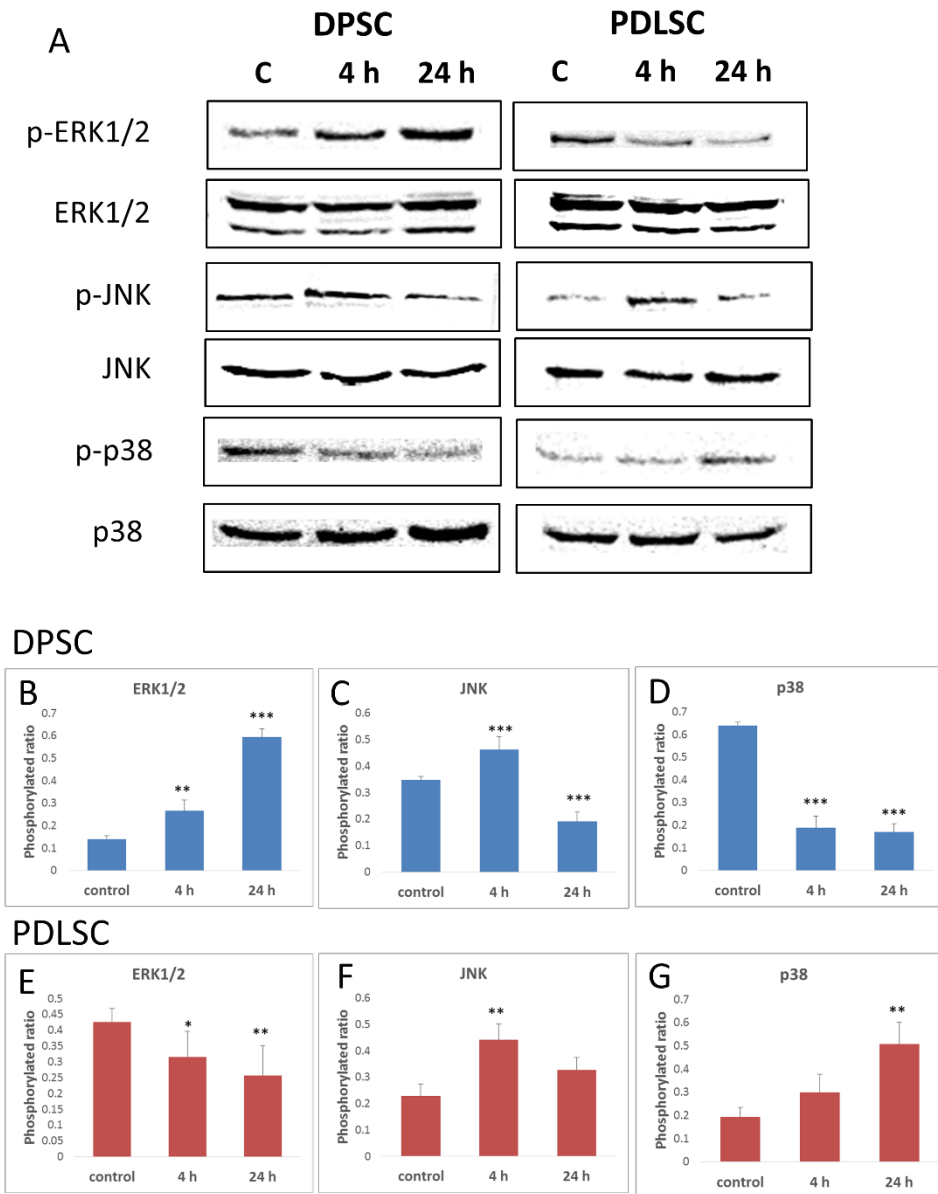
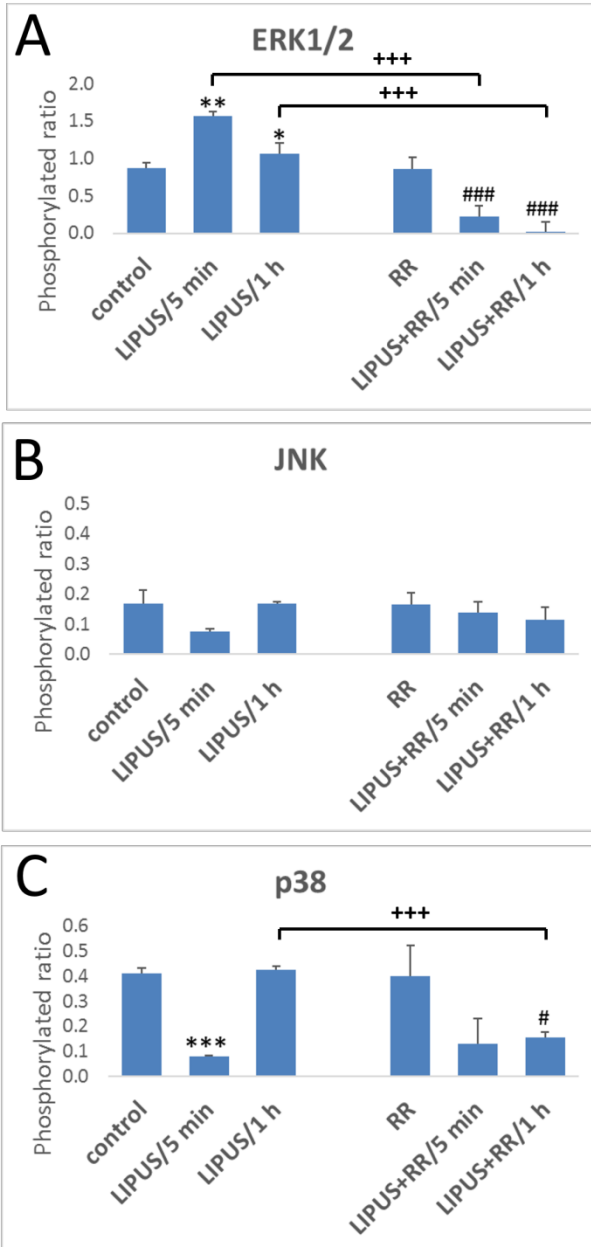


Fig. 3

## DPSC



## PDLSC

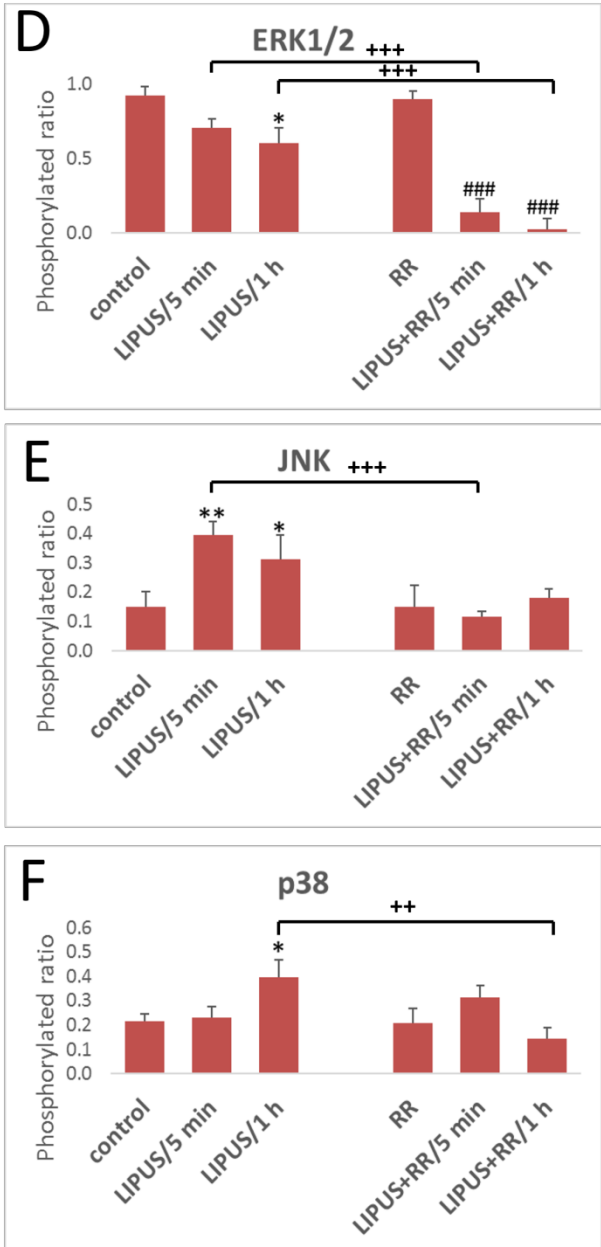


Fig. 4